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14. ABSTRACT

This DOD grant aims to identify biomolecules that are crucial in the pathogenesis of prostate cancer. In the first year we found that miR-125b directly targets p53, Puma and Bak1. In the 2nd year, we focused on Aim 2 and have obtained exciting results: 1) miR-125b promotes prostatic tumor growth and also induces castrate-resistant growth in a mouse model; 2) miR-125-mediated suppression of apoptosis signaling contributes to the growth of CaP cells; and 3) our finding that miR-124 regulates the cellular levels of miR-125b in CaP cells provides a mechanistic explanation for up-regulation of AR/miR-125b signaling in CR CaP cells. Support from this DOD grant has lead to two published papers. One paper addresses the oncogenic activity of miR-125b in CaP cells and the other evaluates the effect of miR-106b on radiation resistance of CaP cells.

15. SUBJECT TERMS

miRNA, tumorigenesis, oncogene, prostate cancer, Bak1, Puma p53

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Table of Contents

Introduction

The subject of the research is "Deregulation of miRNAs contributes to development and progression of prostate cancer". The overall hypothesis of this grant is that *miR-125b* acts as an oncogene, contributing to the development and progression of prostate cancer. In this study, we proposed to test the ability of aberrantly-expressed *miR-125b* to promote tumorigenesis and to induce androgen-independent (AI) growth in three specific aims.

1. To elucidate the mechanism by which *miR-125b* contributes to pathogenesis of prostate cancer. We proposed to validate targets of *miR-125b* in prostate cancer (CaP) and investigate potential signals related to the regulation of *miR-125b*.
2. To evaluate the effects of *miR-125b* on prostatic tumorigenesis and AI growth. We will determine the influence of *miR-125b* on CaP in nude mice.
3. To determine the application of *miR-125b* as a biomarker for prostate cancer. We will detect the abundance of *miR-125b* in clinical prostate samples and determine whether the expression of *miR-125b* correlates to the outcome of patients with CaP.

Here, we reported the completion during the second year.

Body

Research and results

In the 2nd year, our focus is to evaluate the effects of *miR-125b* on prostatic tumorigenesis and AI growth. Besides, we also investigated additional signal pathways that contributes to the regulation of *miR-125b* in CaP. The following studies have been performed and expected results have been obtained.

1. Further evidence showing that miR-125b downregulates p53. We previously found that *miR-125b* directly targets p53 and androgen induces overexpression of endogenous *miR-125b* (**1**). In order to obtain more supportive evidence, we compared androgen-regulated expression of p53 in the parental LNCaP cells and its LNCaP-R273H subline that was previously prepared in our laboratory (**2**). The latter stably expresses a $p53^{R273H}$ mutant allele that does not contain a *miR-125b* binding site due to its lack of the *p53* 3′UTR. In the study, both androgen-receptor (AR)-positive LNCaP and LNCaP-R273H were treated with the synthetic androgen R1881, and p53 protein was detected by Western blot analysis. Consistent with our previous observation (**2**), untreated LNCaP-R273H cells expressed markedly increased p53 compared to the parental LNCaP, while R1881 treatment induced an 80% reduction of p53 in the parental LNCaP cells, but only 40% reduction in LNCaP-R273H cells (**Fig. 1**). Data obtained from this study suggest that R1881-induced *miR-125b* only targeted endogenous wild-type p53 while ectopically-expressed $p53^{R273H}$ evaded regulation.

2. Regulation of miR-125 by miR-124. Based on our previous finding that androgen-independent CaP cells express elevated *miR-125b* and AR signaling regulates *miR-125b* (**1**), we hypothesize that in addition to agonist and antagonist of the AR, biomolecules that modulate the AR expression and activity can alter the cellular abundance of *miR-125b* in CaP cells. We first tested the effect of AR-targeting miRNA on AR. Using TargetScan prediction program, a *miR-124*-binding site was identified in the 3'UTR of *AR* mRNA. We assessed the ability of chemically-modified *miR-124* mimic (miR-124m, purchased from Ambion) to regulate AR expression in the AR-positive LNCaP and C4-2B cells. As shown in **Fig.2A**, transfection of cells with 100 nM of miR-124m induced dramatic reduction of the expression of AR, as well as AR-regulated PSA. Then, we examined whether *miR-124* was able to downregulate *miR-125b*. To address this issue, miR-124m was transfected into two androgen-independent CaP lines, C4-2B and cds2 that highly express the AR and *miR-125b*, and have low levels of *miR-124*. Quantitative PCR analysis of these transfectants revealed that, compared to miR-NC transfectants, enforced expression of *miR-124* induced downregulation of *miR-125b* in both C4-2B and cds2 cell lines [30% ($p<0.05$) and 54% ($p<0.01$) reduction versus control, respectively] (**Fig.2B**). These data strongly suggest that *miR-124* targets the AR/*miR-125b* signaling, regulating the cellular levels of *miR-125b* in CaP cells.

3. miR-125b promotes tumor growth in intact and castrated male nude mice. Our previous *in vitro* experiments demonstrated that *miR-125b* stimulated androgen-independent growth (**1**). In order to assess the influence of *miR-125b* on CaP tumor biology, we developed a PC-346C-*miR-125b* pool for stably overexpress *miR-125b* in androgen-dependent CaP PC-346C cells. PC-346C cell line was selected since it expresses wild-type p53 and wild-type androgen receptor (AR) (**3**) and is androgen-dependent/sensitive (**4**). Furthermore, this cell line has a limited ability to form tumors in intact nude mice and fails to grow in castrated mice (**5**). To do this, a lentiviral *miR-125b* expression vector and a control lentiviral vector were purchased from System Biosciences (SBI, Mountain View, CA). Pseudovirus production and cell transduction were performed following the manufacturer's protocol. The resulting PC-346C-*miR-125b* cells that stably express *miR-125b* were selected through fluorescence-activated cell sorting (FACS). Over-expression of *miR-125b* in infected PC-346C cells was detected using a quantitative RT-PCR approach. We observed a 19-fold increase in *miR-125b* levels in *miR-125b*-infected cells compared to that in control cells (**Fig. 3A**). We also performed soft agar assay of PC-346C-*miR-125b* cells and observed that *miR-125b* treatment resulted in a 3-fold increase in the colony numbers (**Fig. 3B**). Our *in vitro* data indicated that *miR-125b* is able to induce anchorage independent growth (a hallmark of transformed cells) of CaP cells.

We then determined whether *miR-125b* was able to enhance tumorigenicity. Two *in vivo* experiments were performed using the lentiviral-transduced PC-346C-*miR-125b* cells. First, we tested whether *miR-125b* determined tumorigenicity with respect to tumor formation rate and growth kinetics. Intact male nude mice (five mice per group) were injected subcutaneously with PC-346C-*miR-125b* or PC-346C-vector control cells. Tumors arose in all mice injected with PC-346C-*miR-125b* and in four of five control mice. However, PC-346C-*miR-125b* cells produced palpable tumors much faster than control cells with tumors appearing at 7 to 10 days vs. at approximately 21 days, respectively. Furthermore, PC-346C-*miR-125b* tumor volumes at 5 weeks were markedly larger than control tumors (**Fig. 4A**), indicating that in intact mice, elevated *miR-125b* expression accelerates tumor growth kinetics. We next tested whether *miR-125b* could support tumor growth in the absence of androgens. For this, male mice were injected with PC-346C-*miR-125b* cells. When tumors reached ~0.6 cm in the diameter, a subpopulation of mice bearing *miR-125b* tumors were castrated and tumor growth monitored. Castration resulted in a temporary growth regression of *miR-125b* tumors, followed by rapid growth (**Fig. 4B**). These data indicate that *miR-125b* increases the tumorigenicity of and promotes CR growth of PC-346C cells.

4. Inactivation of miR-125b induces apoptosis of CaP cells. Reduced apoptosis can figure prominently in the CR growth of CaP (**6**). Based upon the above finding that *miR-125b* increases the tumorigenicity of prostate cancer and promotes CR growth, we tried to test the hypothesis that downregulation of *miR-125b* activity would result in increased apoptotic cell death. To address the issue, the activity of *miR-125b* in PC-346C cells was antagonized by transfection with anti-*miR-125b* (50 nM). Cells were then cultured in androgen-deprived medium for four days and apoptotic cells detected and quantitated by using a FACS Annexin V assay kit (Trevigen, Inc. Gaithersburg, MD) following the protocol provided by the manufacturer. Data analysis was performed using FACScan software (Becton Dickinson). We observed that in the absence of androgen anti-*miR-125b* induced 30.2% of PC-346C cells to undergo apoptotic cell death (**Fig. 5A**). The comparative figure for the anti-miR-NC cells was 5.4% ($p<0.01$). To provide biochemical evidence for the occurrence of apoptosis, we determined whether downregulation of *miR-125b* increases the release of mitochondrial cytochrome c, SMAC and activated caspase 3. As expected, treatment of PC-346C cells with anti-*miR-125b* induced an enhancement of cytochrome c and SMAC, and a reduction of procaspase 3 (**Fig. 5B**). Therefore, *miR-125b*-mediated suppression of apoptosis signaling contributes to the growth of CaP cells.

Key Research Accomplishments

This DOD grant aims to identify some biomolecule that is crucial in the pathogenesis of prostate cancer. Our previous data have shown that *miR-125b* directly targets p53, Puma and Bak1, and may contribute to development and progression of CaP. In the 2nd year, we have obtained exciting results. The key accomplishments are that ***1***) we have confirmed that *miR-125b* acts as an oncogenic molecule and

promotes prostatic tumor growth in mouse models and also induces castrate-resistant growth of CaP cells; ***2***) we found that *miR-125b*-mediated suppression of apoptosis signaling contributes to the growth of CaP cells, and therefore targeting *miR-125b* may represent a new strategy for CaP treatment; and ***3***) our finding that *miR-124* regulates the cellular levels of *miR-125b* in CaP cells provides a mechanistic explanation for upregulation of AR/*miR-125b* signaling in CR CaP cells.

Reportable Outcomes

Paper publication. Under the support by this grant, we have published two papers. One paper addresses the oncogenic activity of *miR-125b* in CaP cells and the other evaluates the effect of *miR-106b* on radiation resistance of CaP cells.

1. Shi XB, Xue L, Ma AH, Tepper CG, Kung HJ, deVere White RW. *miR-125b* promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes. Prostate, 71:538-549. 2011.
2. Li B, Shi XB, Nori D, Chao CK, Chen AM, Valicenti R, deVere White RW. Down-regulation of microRNA 106b is involved in p21-mediated cell cycle arrest in response to radiation in prostate cancer cells. Prostate, 71:567-574 2011

Published abstract

Shi XB, deVere White RW. Aberrantly expression of *miR-125* promotes growth of prostate cancer. DOD IMPaCT Symposium 2011 (March 10-11 2011, Orlando).

Conclusion

Support from this DOD grant led us to determine that androgens and some non-androgenic factors regulate *miR-125b* and AR/*miR-125b* signaling directly targets three key pro-apoptotic molecules: p53, Puma and Bak1. In the 2nd year, we identified *miR-124* that regulates the AR/*miR-125b* signaling. We also provide evidence showing that aberrantly-expressed *miR-125b* promotes prostatic tumorigenesis and induces castrate resistant growth of CaP cells. Our data strongly suggest that aberrantly expressed *miR-125b* plays an important role in the development and progression of prostate cancer.

In the 3rd year, we will detect the abundance of *miR-125b* in clinical CaP tissues and determine whether the expression level of *miR-125b* correlates to the outcome of patients with CaP.

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6. Cohen MB, Rokhlin OW. Mechanisms of prostate cancer cell survival after inhibition of AR expression. J Cell Biochem 2009*;* 106*(*3*):* 363–371.

Appendices

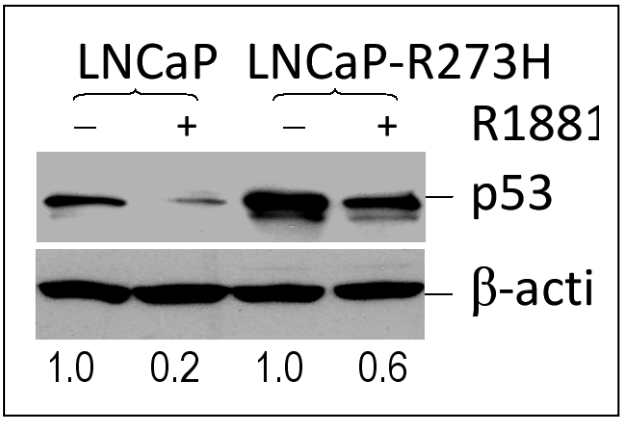

Figure 1. Western blot analysis of p53 protein in 5.0 nM R1881-treated LNCaP cells and LNCaP-R273H cells. β-actin is a loading control, and the underneath numbers are percentages relative to untreated cells, calculated by scanning the p53 bands followed by normalization for β-actin.

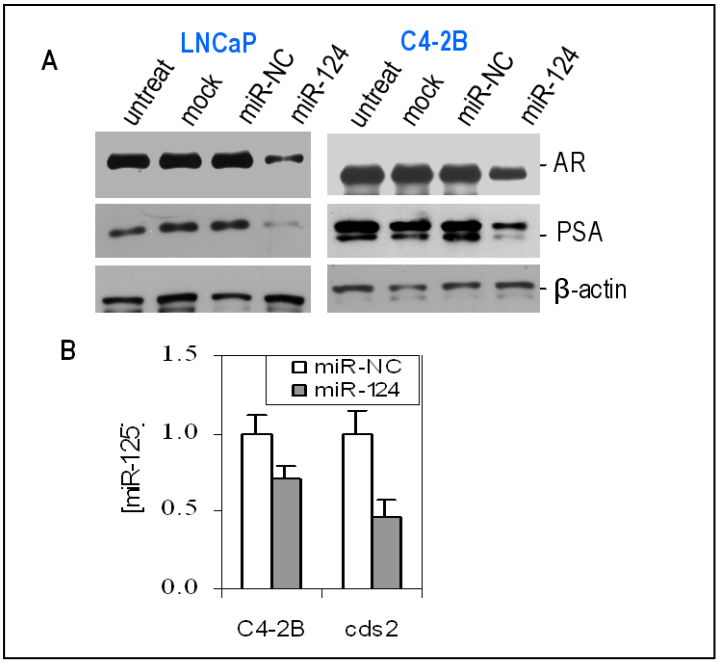

Figure 2. *A*) Western blotting analysis of the expression of AR and PSA in miR-124m-transfected LNCaP and C4-2B. *B*) qPCR analysis of *miR-124*-induced down-regulation of *miR-125*.

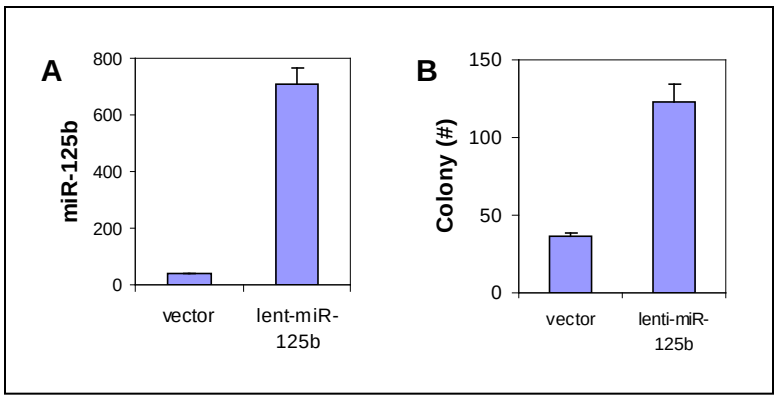

Figure 3. Establishment of lentivirus-infected PC-346C sublines for stable overexpression of *miR-125b*. *A*) Quantitative RT-PCR analysis of *miR-125b* level in infected PC-346C cells. There is a 19-fold increase in *miR-125b* levels in *miR-125b*-infected cells relative to vector control cells. *B*) Soft agar assay of PC-346C-*miR-125b* cells. Lenti-*miR-125b* induces a 3-fold increase in anchorage independent growth.

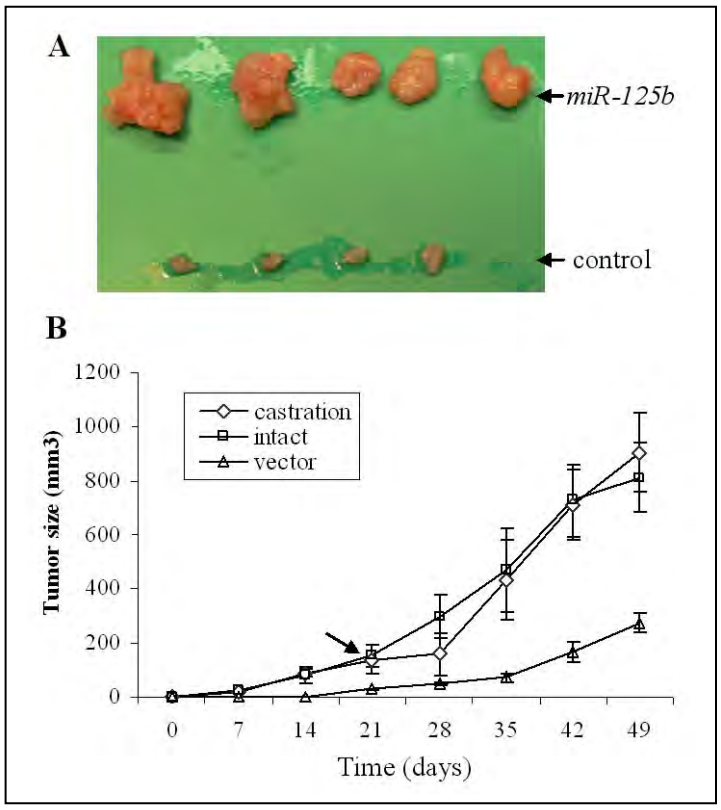

Figure 4. *miR-125b* promotes androgen-dependent and CR growth *in vivo*. **A**: Intact male nude mice (5 per group) each were injected subcutaneously with 2 × 106 PC-346C-lenti-*miR-125b* cells or PC-346C-lenti-vector control cells. Tumors were dissected from *miR-125b* mice (top) and control mice (bottom) 5 weeks after inoculation. **B**: Sixteen intact nude mice each were injected subcutaneously with 2 × 106 PC-346C-lenti-*miR-125b* cells, which overexpress of *miR-125b*. When their tumor size reached approximately ~ 0.5 cm in the diameter, eight of these 16 mice were castrated. The arrow indicates the castration time. Eight intact nude mice were injected subcutaneously with 2 × 106 PC-346C-lenti-vector control cells (vector). Each time point represents mean ± SD of eight independent values.

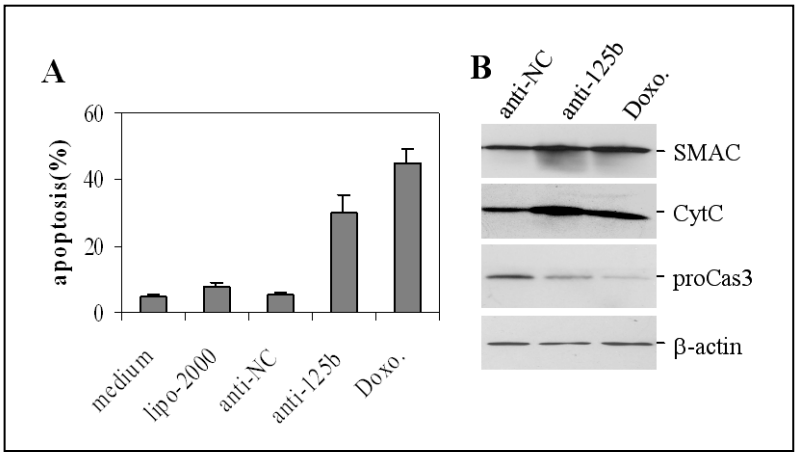

Figure 5. Downregulation of *miR-125b* activity induces apoptosis. **A**: Annexin V assay of apoptosis. PC-346C cells grown in charcoal-deprived medium were treated with 50 nM anti-*miR-125b* (anti-125b) or anti-miRNA negative control (anti-NC) for 4 days and stained with Annexin V and propidium iodide. Both early apoptotic cells (%) and late apoptotic/necrotic cells (%) are combined. Data are means ± SD from three independent experiments. Charcoal-deprived medium (medium), transfection reagent lipofectamine 2000 (lipo-2000), and doxorubicin (Doxo) were the controls. **B**: Western blot analysis of SMAC, cytochrome *c* (CytC), and pro-caspases 3 (proCas3). PC-346C cells were treated with 50 nM anti-*miR-125b* (anti-125b) or anti-miRNA

negative control (anti-NC) for 4 days. The expression levels of proteins were analyzed with specific antibodies. Significant reduction of the pro-caspase 3 band indicates the activation of caspase-3.

miR-125b Promotes Growth of Prostate Cancer Xenograft Tumor Through Targeting Pro-Apoptotic Genes

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BACKGROUND. Increasing evidence demonstrates that aberrantly regulated microRNAs (miRNAs) contribute to the initiation and progression of human cancer. We previously have demonstrated that *miR-125b* stimulated the growth of prostate cancer (CaP) cells. In this study, we further determined the influence of *miR-125b* on the pathogenesis of CaP.
METHODS. To evaluate the effect of *miR-125b* on xenograft tumor growth, male athymic mice were subcutaneously injected with PC-346C-*miR-125b* cells that stably overexpressed *miR-125b*. Potential direct target transcripts of *miR-125b* were identified using a bioinformatics approach and three *miR-125b* targeted molecules were confirmed by means of biochemical analyses.
RESULTS. Enforced expression of *miR-125b* promoted tumor growth in both intact and castrated male nude mice. In an effort to define the molecular mechanism(s) mediating its tumor growth properties, we found that *miR-125b* directly targets eight transcripts, including three key pro-apoptotic genes: p53, Puma, and Bak1. Increasing the abundance of *miR-125b* resulted in a dramatic decrease in the levels of these three proteins in CaP cells. A direct repressive effect on each of these was supported by the ability of *miR-125b* to significantly reduce the activity of luciferase reporters containing their 3′-untranslated regions of each gene encompassing the *miR-125b*-binding sites. Additionally, we found that repression of *miR-125b* activity was able to sensitize CaP cells to different therapeutic interventions.
CONCLUSION. Data obtained in this study demonstrate that *miR-125b* promotes growth of prostatic xenograft tumors by down-regulating three key pro-apoptotic genes. This suggests that miR-125b is oncogenic and makes it an attractive therapeutic target in CaP. *Prostate 71: 538–549, 2011.* © 2010 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; microRNA; p53; pro-apoptotic gene**

INTRODUCTION

Prostate cancer (CaP) is the most frequently diagnosed malignant tumor in males and the second leading cause of cancerous deaths in American men [1]. Patients with metastatic CaP are customarily treated with androgen ablative therapy (AAT). Unfortunately, failure of AAT inevitably occurs and the patient's tumor becomes castration-resistant (CR). At present, no curative treatment is available for CR CaP [2]. Although the precise mechanisms leading to castration resistance are not completely understood, considerable insight into the progression of CaP has been recently achieved. Aberrant expression of some growth/survival-related signal molecules, including their modulator microRNAs (miRNAs), is thought to be one mechanism associated with the development of CR CaP [3].

Conflict of Interest: The authors have declared no conflict of interest.
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Mature miRNAs are a class of 19- to 25-nucleotide RNA molecules. These endogenous small RNAs may negatively regulate up to 30% of the human genes by imperfectly binding to miRNA-binding sites located at the 3′-untranslated regions (3′-UTRs) of targeted genes, causing either degradation of mRNAs or, more commonly for mammalian miRNAs, inhibition of their translation to functional proteins. Thus, miRNAs are involved in cell proliferation, differentiation, stress response, apoptosis, immunity, and transcriptional regulation in many human diseases including cancer [4]. Accumulating evidence supports the potential involvement of altered expression of miRNAs in initiation and progression of a wide range of human cancers, including CaP. It was reported that a number of miRNAs exhibit distinct expression patterns in clinical prostate tumor samples compared to benign prostate tissues [5–8], suggesting that these aberrantly expressed miRNAs are likely implicated in the biology of CaP.

miR-125b is a well-characterized miRNA. In humans, there are two *miR-125b* members: *miR-125b-1* and -2, located on chromosomes 11 and 21, respectively. In CaP cells, the mature *miR-125b* is thought to be derived primarily from *miR-125b-2* [9]. Dysregulation of *miR-125b* has been reported to occur in multiple human cancer types, including cancers of the stomach [10], colon [11], pancreases [12], bladder [13], and ovary [14], as well as in oligodendroglial tumors [15], and acute lymphoblastic leukemia [16]. It was recently reported that highly expressed *miR-125b* confers survival advantage by suppressing apoptosis in acute lymphoblastic leukemia [17]. We previously found that *miR-125b* stimulates androgen-independent growth of CaP cells in vitro [18]. In the current study, we further explored the functional role of *miR-125b* in CaP, and found that enforced expression of *miR-125b* promoted tumor growth in intact and castrated male nude mice. We also performed a series of experiments to investigate the molecular mechanisms accounting for *miR-125b*-mediated growth of CaP cells. The data obtained in this study demonstrate that *miR-125b* has oncogenic properties through its anti-apoptotic and proliferation-promoting activities and suggest that it can be exploited as a therapeutic target in CaP.

MATERIALS AND METHODS

Reagents

Synthetic *miR-125b* mimic (miR-125bm), miRNA negative control (miR-NC), anti-*miR-125b*, anti-*miR*-negative control (anti-miR-NC), and the pMIR-REPORT Luciferase vector were purchased from Ambion. The miR-125bm is short double-stranded RNA oligonucleotide, and it has been chemically modified to increase the stability in vivo and the activity of *miR-125b*. The anti-*miR-125b* was single-stranded RNA oligonucleotides designed to specifically bind to endogenous *miR-125b*, inhibiting its activity but not downregulating its abundance. Genistein combined polysaccharide (GCP) is a nutritional supplement and was generously provided by Amino Up Chemical Company, Ltd. (Sapporo, Japan) and stock solutions were made in 50% DMSO/50% EtOH. Antibodies to Bak1, p21, SMAC, CytC, proCas3 were purchased from Cell Signaling Technology (Danvers, MA) and antibodies to p53 and Puma from Calbiochem.

Cell Lines

CaP cell lines used in this study include PC3, DU145, PC-346C, and LNCaP and its sublines LNCaP-cds2 (named as cds2) and LNCaP-R273H. Both cds2 and LNCaP-R273H were prepared previously in our laboratory [19,20]. PC-346C cell line that was kindly provided by Dr. Adrie van Bokhoven (University of Colorado) was developed from a non-progressive prostate tumor [21]. LNCaP, PC-346C, PC3, and DU145 were routinely maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. For LNCaP-R273H cells, G418 (200 μg/ml) was added to the medium. cds2 cells were cultured in RPMI 1640 medium containing 5% charcoal/dextran-stripped serum (androgen-deprived medium).

Generation of Stable PC-346C-*miR-125b* Population

A lentiviral *miR-125b* expression vector that expresses a ∼500-base pre-*miR-125b* and empty lentiviral vector were purchased from System Biosciences (SBI, Mountain View, CA). Pseudovirus production and cell transduction were performed following the manufacturer's protocol. The resulting PC-346C CaP cells, PC-346C-*miR-125b* that stably overexpress *miR-125b*, were selected through fluorescence-activated cell sorting (FACS) and were maintained in medium described above. Over-expression of *miR-125b* in infected PC-346C cells was detected using a quantitative RT-PCR approach with a *miR-125b*-specific primer (Ambion).

Tumor Xenografts

Male athymic *nu/nu* mice (4- to 6-week-old; Harlan Laboratories) were injected subcutaneously with suspensions of 2×10^6 PC-346C-*miR-125b* or PC-346C-vector control cells made in 50% Matrigel basement membrane matrix (1:1 vol/vol with complete medium; Becton Dickinson). Tumor dimensions were measured

once per week and volumes calculated according to the formula: 1/2 (length × width × height). Castration was performed in a subpopulation of mice when tumors reached a diameter of approximately 0.6 cm. Animal studies were performed according to protocols approved by the Animal Care and Use Committee at University of California, Davis.

Annexin V Binding Assay

Anti-*miR-125b*-induced apoptosis was analyzed using a FACS Annexin V assay kit (Trevigen, Inc., Gaithersburg, MD) following the protocol provided by the manufacturer. Briefly, cells were transfected with anti-*miR-125b* and incubated for 72 hr. The cells were harvested, washed once with PBS, re-suspended in 100 μl of 1× Annexin V binding buffer containing 1 μl of Annexin V-FITC conjugate and 10 μl of propidium iodide solution and then incubated for 30 min at room temperature in the dark. Subsequently, 400 μl 1× Annexin V binding buffer was added and samples analyzed on a FACScan flow cytometer. Data analysis was performed using FACScan software (Becton Dickinson).

Computational Identification of *miR-125b* Target Transcripts

In order to gain insight into the mechanism(s) through which *miR-125b* influences growth and survival of CaP cells, an integrative bioinformatics strategy was developed to identify the target transcripts specifically relevant within the context of our models. This target profiling strategy entailed four steps: (1) defining a *miR-125b*-regulated gene expression profile; (2) generating a comprehensive *miR-125b* target site database by compilation of predicted targets (~943 separate sites) sourced from various publicly-available databases [22,23]; (3) using this database as an index for mining and extracting of the *miR-125b* targets present in our list of differentially expressed genes; and (4) selecting candidate genes based upon gene ontology and pathway analyses. For the first component, the list of *miR-125b*-regulated genes in LNCaP cells was obtained by overexpression of *miR-125b* followed by Affymetrix gene expression profiling and comparison analysis for differentially expressed genes with DNA-Chip analyzer (dChip) software [24]. Extraction of predicted *miR-125b* target transcripts from the expression profile was accomplished with an Excel add-in ''parsing'' tool from the Macros for Microarray (MfM) software (UC Davis Cancer Center Genomics and Expression Resource). Since miRNAs can negatively regulate the genes via accelerated decay, the resulting *miR-125b* target profile was limited to the down-regulated transcripts. Using the GO terms appended to each entity, the genes were then filtered based upon function in biological processes including apoptosis, tumor suppression, and metastasis.

Western Blot Analysis

Cells were grown to 70–80% confluence and lysed using the cell lysis buffer (Cell Signaling Technology; Cat#: 9803). Denatured protein samples (20–100 μg) were resolved on 10% SDS–polyacrylamide gels, and then transferred to Immobilon PVDF membrane (Millipore). After blocking with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween 20 (TBST), the membrane was incubated with a specific primary antibody, followed by the horseradixh peroxidase-conjugated secondary antibody. Proteins were visualized using ECL reagents.

Reporter Constructs and Luciferase Assay

To construct reporter plasmids containing the 3′-DNA regions of *p53* and *PUMA*, a 0.5-kb DNA fragment of the *p53* 3′-UTR and a 0.85-kb DNA fragment of the *PUMA* 3′-UTR that contain the putative *miR-125* binding sites were prepared. Negative control plasmids containing 0.4-kb *p53* and 0.7-kb *PUMA* 3′-UTR fragments that lack the *miR-125b* binding sites were also prepared. DNA fragments were cloned into the pMIR-REPORT Luciferase vector downstream of the reporter gene. The sequences and cloning direction of these PCR products were validated by DNA sequencing. For luciferase assay, cells (4×10^4 per well) were seeded into 24-well plates and cultured for 24 hr. The cells were then transfected with reporter plasmids. The pRL-SV40 *Renilla* luciferase plasmid (Promega) was used as an internal control. Two days later, cells were harvested and lysed with passive lysis buffer (Promega). Luciferase activity was measured using a dual luciferase reporter assay (Promega). The results were expressed as relative luciferase activity (Luc/*Renilla*).

Evaluation of *miR-125b* Antagonism on Growth With WST-1 Assay

Cells (4.5×10^3 per well) were plated in 96-well plates in androgen-deprived medium. After being cultured for 24 hr, cells were transfected with anti-*miR-125b* or anti-miR-NC at a concentration of 50 nM using lipofectamine 2000 (Invitrogen). Five hours later, cells were treated with fresh medium or, if necessary, fresh medium containing anti-CaP drug in optimized concentration. The transfection protocol was optimized using a fluorescent pEGFP-N1 vector (Clontech), and resulted in the transfection of >90% of cds2 cells. Tetrazolium-based cell proliferation assay (WST-1;

Promega) was then carried out at various days after transfection according to the manufacturer's protocol.

RESULTS

miR-125b Promotes Tumor Growth in Intact and Castrated Male Nude Mice

Our previous in vitro experiments demonstrated that *miR-125b* stimulated androgen-independent growth [18]. In this study, we determined whether *miR-125b* was able to enhance tumorigenicity. For this purpose, we generated a stable PC-346C-*miR-125b* pool by infection with a *miR-125b*-expressing lentiviral vector. Quantitative RT-PCR analysis of *miR-125b* abundance exhibited a 19-fold increase in *miR-125b* levels in *miR-125b*-infected cells compared to that in control cells (data not shown). PC-346C cell line was selected since it expresses wild-type p53 and wild-type AR [25] and is androgen-dependent/sensitive [26]. Furthermore, this cell line has a limited ability to form tumors in intact nude mice and fails to grow in castrated mice [27]. Two in vivo experiments were performed using the lentiviral-transduced PC-346C-*miR-125b* cells. First, we tested whether *miR-125b* determined tumorigenicity with respect to tumor formation rate and growth kinetics. Intact male nude mice (five mice per group) were injected subcutaneously with PC-346C-*miR-125b* or PC-346C-vector control cells. Tumors arose in all mice injected with PC-346C-*miR-125b* and in four of five control mice. However, PC-346C-*miR-125b* cells produced palpable tumors much faster than control cells with tumors appearing at 7–10 days versus at approximately 21 days, respectively. Furthermore, PC-346C-*miR-125b* tumor volumes at 5 weeks were markedly larger than control tumors (Fig. 1A), indicating that in intact mice, elevated *miR-125b* expression accelerates tumor growth kinetics. We next tested whether *miR-125b* could support tumor growth in the absence of androgens. For this, male mice were injected with PC-346C-*miR-125b* cells. When tumors reached ~0.6 cm in the diameter, a subpopulation of mice bearing *miR-125b* tumors were castrated and tumor growth monitored. Castration resulted in a temporary growth regression of *miR-125b* tumors, followed by rapid growth (Fig. 1B). In summary, these data indicate that *miR-125b* increases the tumorigenicity of and promotes CR growth of PC-346C cells.

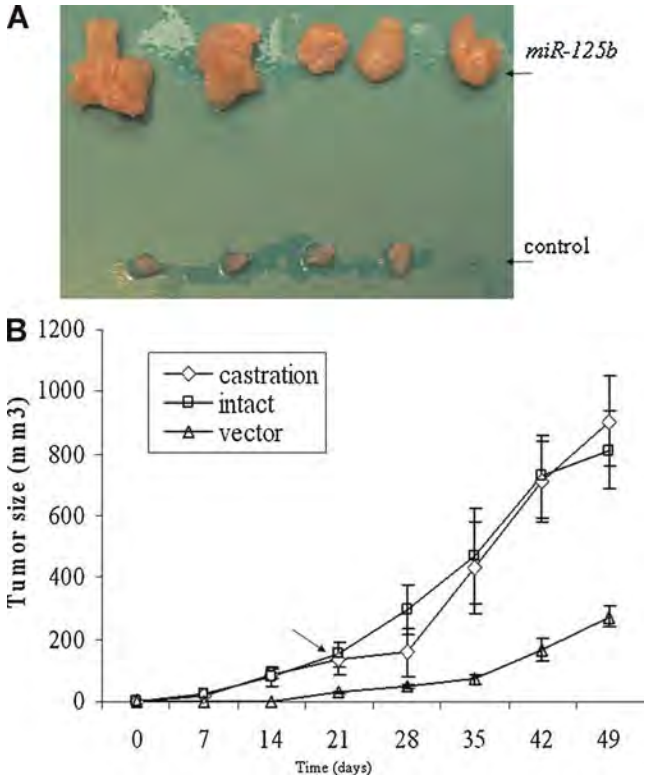


Fig. 1. *miR-125b* promotes androgen-dependent and CR growth in vivo. **A**: Intact male nude mice (5 per group) each were injected subcutaneously with 2×10^6 PC-346C-*miR-125b* cells or PC-346C-vector control cells. Tumors were dissected from *miR-125b* mice (top) and control mice (bottom) 5 weeks after inoculation. **B**: Sixteen intact nude mice each were injected subcutaneously with 2×10^6 PC-346C-*miR-125b* cells, which overexpress of *miR-125b*. When their tumor size reached approximately 0.125 cm^3 (~0.6 cm in the diameter), eight of these 16 mice were castrated. The arrow indicates the castration time. Eight intact nude mice were injected subcutaneously with 2×10^6 PC-346C-vector control cells (vector). Each time point represents mean ± SD of eight independent values.

Inactivation of *miR-125b* Induces Apoptosis of CaP Cells

Reduced apoptosis can figure prominently in the CR growth of CaP [28]. Based upon the results above, we wanted to test the hypothesis that downregulation of *miR-125b* activity would result in increased apoptotic cell death. To address this, the activity of *miR-125b* in PC-346C cells was antagonized by transfection with anti-*miR-125b* (50 nM). Cells were then cultured in androgen-deprived medium for 4 days and apoptotic cells detected and quantitated by Annexin V binding assay. We observed that in the absence of androgen anti-*miR-125b* induced 30.2% of PC-346C cells to undergo apoptotic cell death (Fig. 2A). The comparative figure for the anti-miR-NC cells was 5.4% ($P < 0.01$). To provide biochemical evidence for the occurrence of apoptosis, we determined whether downregulation of *miR-125b* increases the release of mitochondrial cytochrome *c*, SMAC and activated caspase 3. As expected, treatment of PC-346C cells with anti-*miR-125b* induced an enhancement of cyto-

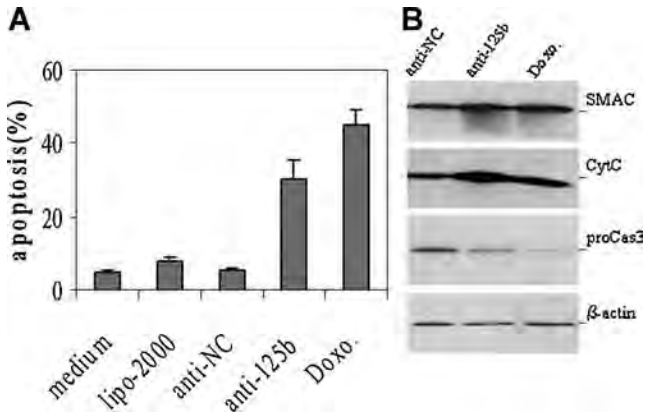


Fig. 2. Downregulation of *miR-125b* activity induces apoptosis. **A**: Annexin V assay of apoptosis. PC-346C cells grown in charcoal-deprived medium were treated with 50 nM anti-*miR-125b* (anti-125b) or anti-miRNA negative control (anti-NC) for 4 days and stained with Annexin V and propidium iodide. Both early apoptotic cells (%) and late apoptotic/necrotic cells (%) are combined. Data are means ± SD from three independent experiments. Charcoal-deprived medium (medium), transfection reagent lipofectamine 2000 (lipo-2000), and doxorubicin (Doxo) were the controls. **B**: Western blot analysis of SMAC, cytochrome *c* (CytC), and pro-caspases 3 (proCas3). PC-346C cells were treated with 50 nM anti-*miR-125b* (anti-125b) or anti-miRNA negative control (anti-NC) for 4 days. The expression levels of proteins were analyzed with specific antibodies. Significant reduction of the pro-caspase 3 band indicates the activation of caspase-3 [49].

chrome *c* and SMAC, and a reduction of procaspase 3 (Fig. 2B). Therefore, *miR-125b*-mediated suppression of apoptosis signaling contributes to the growth of CaP cells.

Identification of *miR-125b* Targets

In order to better define the molecular mechanisms by which *miR-125b* promotes CaP cell growth and modulates apoptosis, we utilized a bioinformatics approach to identify its potential targets. For this, we compiled a database of the computationally predicted *miR-125b* target sites (from MSKCC) and then used it as an index to mine a gene expression profile composed of transcripts that were differentially expressed as a result of transient overexpression of *miR-125b* in CaP cells. In this manner, candidate direct targets are extracted from the entire dataset, which more than likely also contained transcripts regulated as a secondary response. The resulting putative, CaP-relevant *miR-125b* targets were then classified based upon Gene Ontology terms in order to determine the mRNAs whose suppression was most likely responsible for the alteration in one or more biological processes, which was described in Materials and Methods section. In addition to Bak1, which we previously confirmed and characterized as a *miR-125b* target, seven additional genes were identified that contain *miR-125b*-binding sites in their 3′-UTRs (Table I). Of these, three pro-apoptotic molecules (p53, Puma and Bak1) that are functionally inactivated either by mutation and/or dysregulation, have been reported to play important roles in the initiation and progression of CaP. The other five genes are potential tumor or metastasis suppressors in other cancer types. Myotubularin-related protein 3 (*MTMR3*) induces the expression of p27 [29]; BRCA1-associated protein-1 (*BAP1*) interacts with BRCA1 and suppresses cancer cell growth [30]; sphingosine-1-phosphate lyase 1 (*SGPL1*) may play a role in cancer-surveillance pathways [31]; aryl-hydrocarbon receptor repressor (*AHRR*) is an angiogenic factor [32]; and sialidase 1 (lysosomal sialidase; *NEU1*) inhibits tumor metastasis by decreasing integrin β4 signaling and down-regulating MMP-7 [33]. However, their involvement in CaP remains largely unknown.

miR-125b Down-Regulates Pro-Apoptotic Molecules

Several approaches were then used to confirm that these three pro-apoptotic genes are *bona fide* targets

TABLE I. Genes Directly Targeted by *miR-125b* in LNCaP Cells

Name	Fold change[a]	Function	*miR-125b*-binding site at 3′-UTR
p53	−1.3	Tumor suppressor	733–739
Puma	−2.5	Pro-apoptosis	24–30
Bak1	−2.0	Pro-apoptosis	630–637
Mtmr3	−1.4	Tumor suppressor	941–947
Bap1	−1.6	Tumor suppressor	580–586
Sgpl1	−1.9	Tumor suppressor	1586–1592
Ahrr	−1.5	Tumor suppressor	1275–1281
Neu1	−1.7	Metastasis suppressor	331–337

[a]Fold change: signal in *miR-125* treated cells/that in miR-NC-treated cells.

of *miR-125b*. First, Western blot analysis of lentiviral-transduced PC-346C-*miR-125b* tumor cell lysates demonstrated that *miR-125b* overexpression markedly reduced the protein levels of p53, Puma and Bak1 (left panel in Fig. 3A). Similar results were observed (right panel in Fig. 3A) in cds2 cells that express fivefold greater *miR-125b* than parental LNCaP cells do [18]. Next, we determined whether elevating *miR-125b* levels ectopically with a chemically modified *miR-125b* mimic (miR-125bm) would down-regulate these pro-apoptotic molecules in untreated or irradiated LNCaP cells. As expected, irradiation significantly enhanced the cellular level of p53 and its two direct effectors, p21 and Puma. Treatment with miR-125bm resulted in a marked decrease in both basal and irradiation-induced p53 levels (Fig. 3B). Moreover, downregulation of p53 by miR-125bm also reduced the expression of its effectors p21 and Puma. Consistent with our previous observation [18], miR-125bm targets Bak1. Additionally, we performed immunocytochemistry using enzymatic approaches to stain both p53 and Puma in LNCaP cells that had been transfected with miR-125bm and then irradiated. Similar to the data obtained from Western blot analysis, ectopic expression of miR-125bm induced a marked downregulation of p53, Puma, and Bak1 (Fig. 3C).

Mechanistic analysis of *miR-125b* as a direct regulator of Puma expression in the context of p53-expressing CaP cells is complicated by the fact that Puma is a transcriptional target of p53, which is also a target of *miR-125b*. Thus, it is difficult to discern whether the downregulation of Puma is due to a direct effect of *miR-125b* or to the decrease of p53 caused by *miR-125b*. To validate that *miR-125b* directly targets Puma, p53-null PC3 cells were treated with miR-125bm followed by treatment with resveratrol (100 μM), which was previously reported to upregulate Puma and Bak1 in PC3 cells [34]. Western blot analysis confirmed that transfection with miR-125bm resulted in obvious reductions in resveratrol-stimulated Puma and Bak1 expression, respectively (Fig. 3D). Taken together, these data strongly support that *miR-125b* down-regulates the expression of p53, Puma, and Bak1 in CaP cells.

Androgen Down-Regulates Pro-Apoptosis Molecules

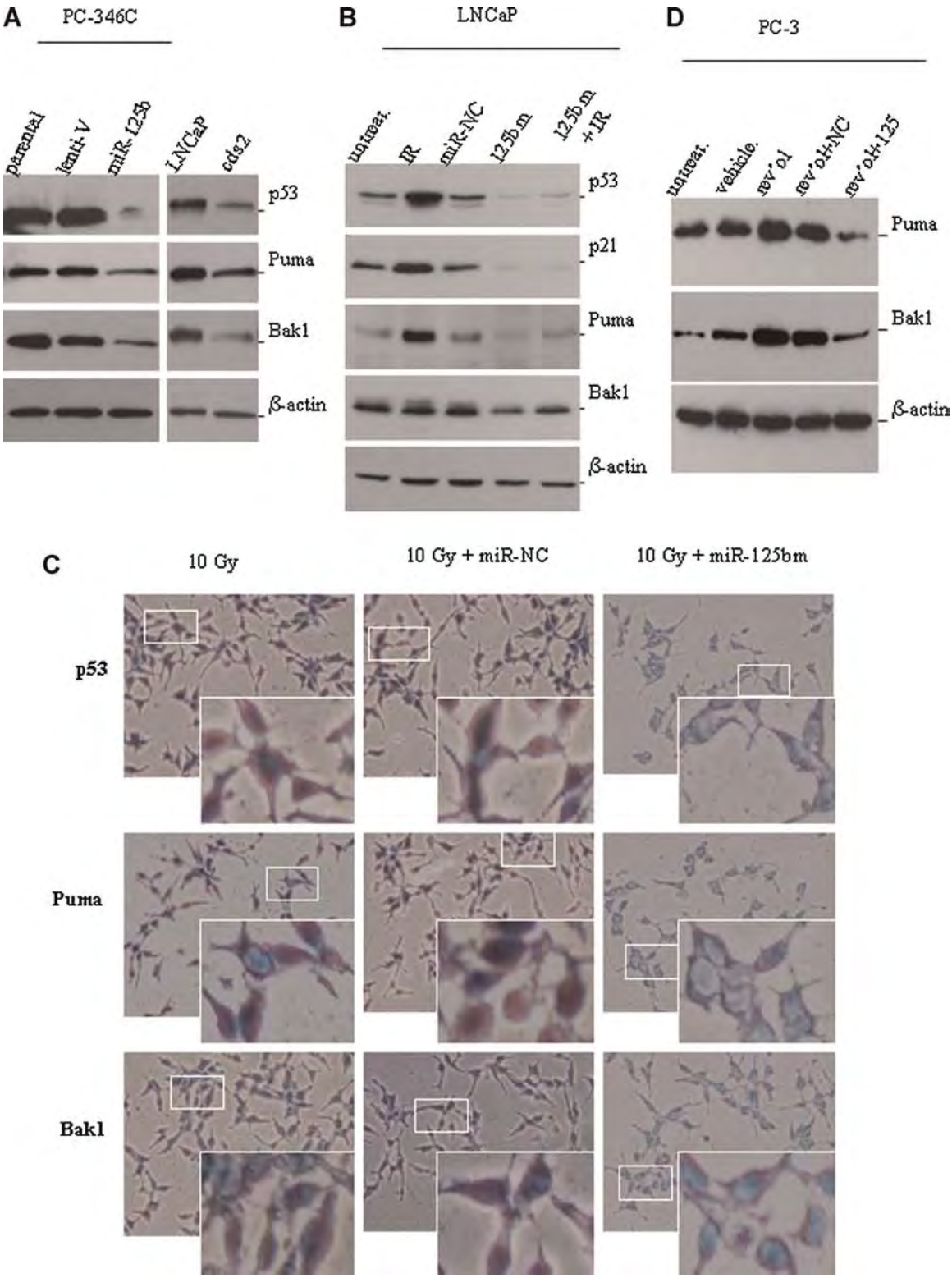
As presented in our previous reporter [18], androgen upregulates the expression of *miR-125b* and consequently resulted in a reduction of Bak1 levels, we thus tested whether androgen treatment decreases the levels of p53 and Puma as well. To address this issue, LNCaP cells were treated with the synthetic androgen R1881 and Western blot analysis was performed to determine the levels of p53 and Puma, as well as Bak1 as a positive control. Similar to the previous observation, Bak1 expression was down-regulated by androgen and up-regulated by anti-*miR-125b*. When compared to untreated and vehicle-treated cells, R1881 induced a marked reduction of basal p53 and Puma levels (Fig. 4A). LNCaP cells were also treated with anti-*miR-125b* to decrease the activity of *miR-125b*. We found that ectopic expression of anti-*miR-125* increased the expression of p53 and Puma proteins by approximately 2.0-fold (Fig. 4A), which was quantitatively measured using a Molecular Imager. In addition, when LNCaP cells were pre-treated with R1881 for 48 hr prior to irradiation with 10 Gys to stimulate increased expression of p53, it was found that R1881 treatment resulted in a 71% and 62% reduction of irradiation-stimulated up-regulation of p53 and Puma, respectively (Fig. 4B).

miR-125b Binds to the 3′-UTRs of *p53* and *PUMA*

Mature miRNAs negatively regulate gene expression by binding to the 3′-UTRs of target transcripts mediated by the complementarity of their 5′-end seed sequences (i.e., nucleotides 2–8 or 2–7) with miRNA-binding site sequences [35,36]. We previously reported that *miR-125b* can bind to the 3′-UTR of BAK1 transcript that contains a binding site for miR-125b [18]. Using the

Fig. 3. *miR-125b* targets pro-apoptotic genes. **A**: Western blot analyses of the expression levels of p53, Puma, and Bak1 in lenti-*miR-125b*-infected PC-346C cells (left panel), and in LNCaP cells grown in 10% FBS medium and in cds2 cells grown in androgen-deprived medium (right panel). Lent-V, lentiviral vector. **B**: Western blot analysis of the expression of p53, p21, Puma, and Bak1 in miR-125bm-treated LNCaP cells. Cells were first transfected with 50 nM of chemically modified miR-125b mimic (125bm) and 24 hr later irradiated with 10 Gys (IR). Eight hours after irradiation, cells were lysed and protein was extracted for Western blot analysis of p53, p21, Puma, and Bak1. The controls include untreated cells (untreat.) and miRNA negative control (miR-NC)-treated cells. **C**: Detection of the expression levels of p53, Puma, and Bak1 in LNCaP cells by immunostaining. LNCaP cells were grown for 24 hr on sterile slides in 100 -mm Petri dishes in 10% FBS medium. Cells were first transfected with 50 nM of chemically modified miR-125bm and 24 hr later irradiated with 10 Gys. Eight hours later, cells were fixed. p53, Puma, or Bak1 were stained using specific 1st antibodies followed by a HRP-labeled 2nd antibody. Then, addition of the substrate (DAB) led to generate the brown color. The enzymatic approach shows that p53 locates mainly in nuclei, and Puma and Bak1 in cytoplasm. The white squares indicate the areas of magnified images. **D**: PC3 cells were transfected with 50 nM of miR-125bm (125bm) and 24 hr later treated with 100 μM resveratrol. Cells were lysed next day and protein was isolated for Western blot analysis of Puma and Bak1. Untreated cells (untreat.) and vehicle-treated cells are used as controls. β-actin is a loading control.

TargetScan program, we identified the *miR-125b* binding sites in the 3′-UTR regions of both *p53* and *PUMA*. To determine whether these putative *miR-125b* binding sites are responsible for the negative post-transcriptional regulation of *p53* and *PUMA* by *miR-125b*, pMIR-REPORT luciferase reporter constructs containing the 3′-UTR fragments of *p53* or *PUMA* were co-transfected with miR-125bm into the *miR-125b*-null DU145 cells. As shown in Figure 5A, cotransfection resulted in a 57% and 45% reduction of the reporter activity for *p53* and



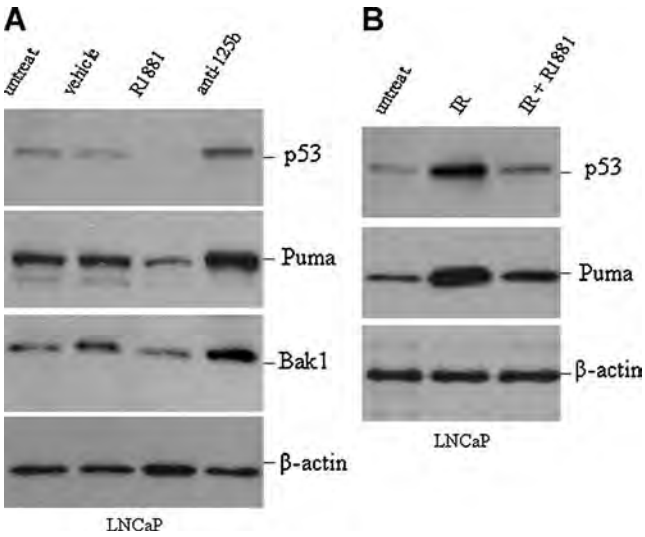


Fig. 4. Effect of androgen on the expression of p53, Puma, and Bakl. **A**: LNCaP cells were treated with 5.0 nM of R1881 or 100 nM anti-miR-125b (anti-125b) for 48 hr. Subsequently, cells were harvested for Western blot analysis of p53, Puma, and Bakl. **B**: LNCaP cells were treated with 5.0 nM R1881 for 48 hr followed by 10 Gys of irradiation (IR). Eight hours after irradiation, the cellular levels of p53 and Puma were analyzed by Western blotting. Controls include untreated cells (untreat.) and ethanol vehicle. β-actin is a loading control.

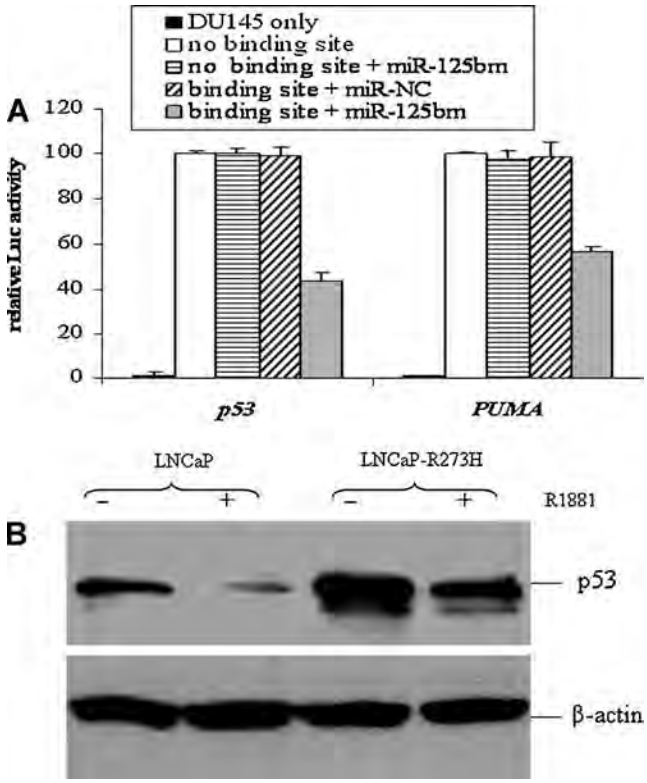


Fig. 5. *MiR-125b* binds to the 3′-UTRs of *p53* and *PUMA*. **A**: Luciferase analysis of the 3′-UTRs of *p53* and *PUMA* in DU145 cells. Individual truncated 3′-UTRs of *p53* and *PUMA* were used as controls (no binding site) in these experiments. The assay was repeated three times with each assay being performed in three wells and similar results were obtained each time. The representative results are shown as mean ± SD (n = 3). **B**: Western blot analysis of p53 protein in 5.0 nM R1881-treated LNCaP cells and LNCaP-R273H cells.

PUMA, respectively. As a complimentary approach, we compared the androgen-regulated expression of p53 protein in the parental LNCaP cell line and its LNCaP-R273H subline. The latter ectopically expresses a $p53^{R273H}$ mutant allele from a cDNA expression cassette that does not contain a *miR-125b* binding site due to its lack of the *p53* 3′-UTR. Consistent with the previous observation, LNCaP-R273H cells expressed markedly increased p53 compared to the parental LNCaP [20]. R1881 treatment induced an 78% reduction of p53 in the parental LNCaP cells, but only 40% reduction in LNCaP-R273H cells (Fig. 5B), suggesting that R1881-induced *miR-125b* only targeted endogenous wild-type p53 while ectopically expressed $p53^{R273H}$ evaded regulation. Taken together, these results provide experimental validation that the 3′-UTRs of p53 and *PUMA* are targets of *miR-125b*.

Down-Regulation of *miR-125b* Sensitizes CaP Cells to Anti-Cancer Drugs

Our data presented above and in previous report [18] demonstrated that downregulation of *miR-125b* abundance inhibits the growth of CaP cells. We thus asked whether this miRNA is a potential therapeutic target that would improve the efficiency of existing CaP therapy. To address this issue, we evaluated the effect of a combination of anti-*miR-125b* and an anti-CaP drug on the growth inhibition or apoptosis of CaP cells. We have previously reported that GCP, an isoflavone-enriched nutriceutical, lowers the threshold at which CaP cells undergo apoptosis; hence, it was used here. To verify that the combination would increase the growth inhibition, GCP and anti-*miR-125b* were used to treat androgen-independent cds2 cells that overexpress natural *miR-125b* [18]. We observed that the combined treatment resulted in a significant inhibition ($P < 0.05$) of the CR growth compared to treatment with a single chemical (Fig. 6A). Additionally, we also tested cisplatin as it is a commonly used chemotherapy in the treatment of solid malignancies including CaP. We used this combinational strategy to treat PC-346C cells that were cultured in medium containing 10% FBS and evaluated the extent to which they underwent apoptosis. It was found that anti-*miR-125b* plus 5 μM cisplatin increased the number of CaP cells that entered apoptosis in the presence of androgens ($P < 0.05$, Fig. 6B). The results suggest that down-regulation of

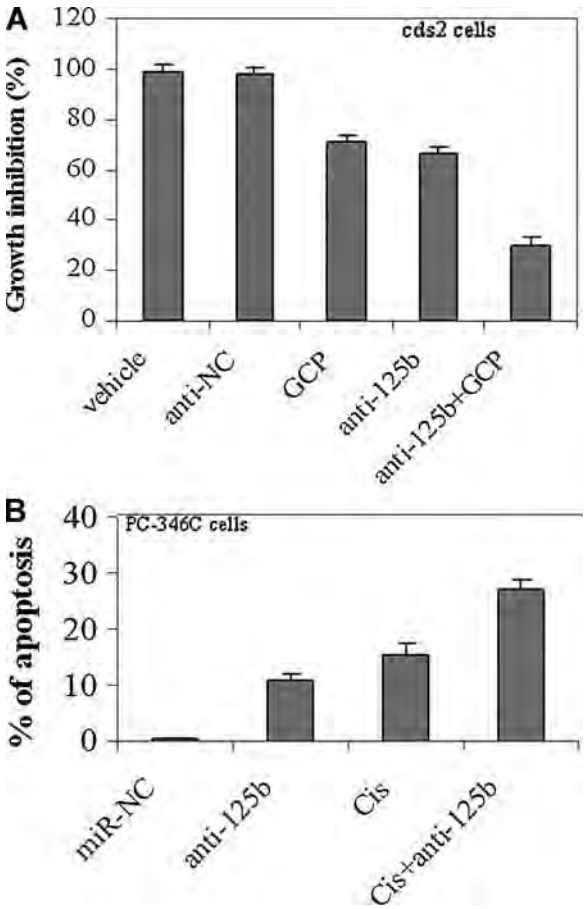


Fig. 6. Effect of down-regulation of *miR-125b* on anti-cancer drugs. **A**: WST-1 assay of growth inhibition. CR cds2 cells were grown in androgen-deprived medium and treated for 5 days using GCP (75 μg/ml) and anti-*miR-125b* (anti-125b, 50 nM) in alone or in a combination. The controls include both vehicle and anti-miRNA-negative control (anti-NC). WST-1 analysis was used to measure the cell growth. The results are expressed as the growth inhibition relative to that treated with vehicle (100%), and shown as mean ± SD (n = 4). **B**: Annexin V assay of apoptosis. PC-346C cells cultured in medium containing 10% FBS were treated using Cisplatin (Cis, 5 μM) and anti-*miR-125b* (anti-125b, 50 nM) for 2 days in alone or in a combination. Cells were harvested and analyzed on the FACScan flow cytometer. Both early apoptotic cells (%) and late apoptotic/necrotic cells (%) are combined. Data are means ± SD from three independent experiments.

miR-125b level can increase the sensitivity of CaP cells to different treatments.

DISCUSSION

This study extends our previous observation that *miR-125b* stimulates CR growth in vitro [18]. In the present study, we demonstrated that *miR-125b* enhances tumorigenicity of CaP cells in intact and castrated mice. Furthermore, we found that *miR-125b* mediates a broad attenuation of the intrinsic apoptosis pathway by targeting p53, Puma, and Bak1. Although an early investigation reported that *miR-125b* was down-regulated in five of nine clinical CaP tissues related to four benign prostate samples [6], several later studies demonstrated that clinical CaP samples over-expressed *miR-125b*. Prueitt et al. [8] detected a 4.29-fold increase in *miR-125b* in locally invasive tumors compared to those without local invasion. Mitchell et al. [37] observed a 6.35-fold increase in circulating *miR-125b* in metastatic CaP compared to that found in sera from healthy men. We previously reported that prostate tumors with high Gleason scores overexpressed *miR-125b* [18]. Our experimental results obtained in the present study, together with a majority of previous clinical data, strongly suggest that aberrantly expressed *miR-125b* has attributes of an oncogene by virtue of promoting the development and progression of CaP. Moreover, we found that androgen withdrawal by lowering *miR-125b* leads to an increase in p53 expression, which is consistent with Agus's observation that androgen withdrawal induced expression of p53 [38]. Our finding provides a molecular rationale for why androgen withdrawal may improve the outcome for CaP patients who undergo radiation therapy. Additionally, we found that repression of *miR-125b* activity sensitizes CaP cells to undergo apoptosis after treatment with various therapies, suggesting that *miR-125b* may be exploited as a target for CaP therapy.

While *miR-125b* has been reported to be highly expressed in many types of human cancers, including CaP, it remains to be determined how *miR-125b* affects the growth of CaP cells. At the initiation of the study, like most of miRNAs, the precise physiologic targets of *miR-125b* had not been validated experimentally. We reasoned that for *miR-125b* to be a driver miRNA, it would need to satisfy the criteria of targeting relevant and potent effector molecules in the context of CaP cells. In our earlier report, Bak1 was identified as a target of *miR-125b*. In the present study, p53 and Puma were further validated to be direct targets of *miR-125b*, which is consistent with a recent finding that *miR-125b* is an important negative regulator of p53 [39]. Since p53, Puma and Bak1 are pro-apoptotic molecules, our results show that *miR-125b* is involved in the p53 network and plays a role in how it regulates apoptosis. Conversely, the data provide a mechanistic explanation for why suppression of *miR-125b* can inhibit tumor growth. In addition to these three pro-apoptotic molecules, *miR-125b* also targets five additional tumor suppressor mRNAs. The precise roles of these putative targets in CaP are unknown. It is possible that decreased expression of these tumor suppressors, together with down-regulated pro-apoptotic molecules, contributes to tumorigenesis and progression of CaP by providing the cells a survival advantage in an

androgen-sufficient or androgen-deprived environment. In the ongoing studies, we will conduct in vitro and in vivo experiments to evaluate the effects of individual molecules on the growth of CaP cells. Elucidation of their contribution to CaP will shed light on understanding mechanism underlying the tumorigenesis and progression of CaP, which might result in identify new molecular targets that would be used to develop potential treatments for this disease.

Data obtained from this study show that *miR-125b* is involved in the governance of proliferation and apoptosis in CaP. Under normal conditions, pro-apoptotic molecules induce mitochondrial outer membrane permeability, causing the release of apoptotic molecules (e.g., cytochrome *c*), subsequent caspase activation, and ultimately leading to apoptotic cell death. In contrast, anti-apoptotic molecules promote survival by antagonizing apoptotic mediators at one or more levels. In CaP cells, particularly in CR tumors, growth is determined by the relative balance between cell proliferation and cell death (apoptosis). Aberrantly expressed *miR-125b* down-regulates p53, Puma, and Bak1. As critical regulators of apoptosis, these molecules have been shown to play a crucial role in suppression of the malignant phenotype of CaP cells [40–42]. Therefore, aberrant *miR-125b* expression resets the balance of apoptosis by directly targeting these molecules and indirectly affecting other members of the Bcl-2 family, conferring a survival advantage to CaP cells. Additionally, it is well known that the AR plays a dominant role in both the initiation and progression of CaP. Studies have demonstrated that wild-type p53 can repress AR activity [43]. We observed that the AR signaling up-regulates *miR-125b* and *miR-125b* in turn down-regulates wild-type p53. We thus speculate that this is a self-sustaining loop in CaP cells, which positively regulates the growth of CaP cells.

We, as well as others [39], validated that *miR-125b* is a direct, negative regulator of wild-type p53 by suppressing its basal expression and up-regulation in response to pro-apoptotic stimuli. Here, we asked how aberrant *miR-125b* expression affects apoptosis in CaP cells harboring a *p53* mutation Addressing this issue is of clinical importance, since mutation of *p53* occurs in more than 40% of CaPs [44–46]. At present, the exact answer to this question has not been determined. Data obtained from this present study provides some insight regarding repression of apoptosis by *miR-125b* in p53-inactivated CaP cells, which may involve targeting Puma and Bak1. In addition to p53 that directly regulates Puma, some of the other cell death and survival signals have been reported to upregulate Puma in a p53-independent fashion [47,48]. Consistent with a previous report [34], we also observed that resveratrol induces an increase in Puma and Bak1 in p53-null PC3 CaP cells. Furthermore, increased *miR-125b* was able to significantly reduce resveratrol-stimulated Puma and Bak1. Therefore, *miR-125b*-mediated downregulation of Puma and Bak1 may play a key role in the p53-inactivated cases. Indeed, one study demonstrated that low Puma expression was associated with a rapid biochemical recurrence of CaP [41].

Given the complexity implicated in the castration resistance of CaP, treatment of this disease may rely on multimodal combination regimens. Until recently, however, chemotherapy has demonstrated only a small survival benefit in patients with CR CaP. The current strategy regarding the treatment of this disease is to prevent occurrence of castration resistance. The fact that one miRNA may target a large number of coding genes involving diverse signaling pathways make the non-encoding small RNA attractive in the design of combination therapy regimens. Indeed, recent study showed that molecular alterations elicited by targeting single miRNAs can provide growth advantages to tumor cells [17]. Aberrant expression of *miR-125b* occurs in at least a subset of CaP cases. This miRNA targets various molecules related to the progression and therapy-resistance of CaP, which implicates it as an emerging alternative treatment option. In this study, we presented evidence that *miR-125b* has oncogenic function. It stimulates CaP growth in xenografts through an anti-apoptotic pathway, very importantly in both the presence and absence of androgens. Therefore, beyond CR CaP, this miRNA also is important in CaP progression. The anti-apoptotic phenotype was not an artifact of *miR-125b* overexpression in CaP cells. Its relevance to CaP cells is suggested by the sensitization to apoptosis induced by GCP after knockdown of the endogenous *miR-125b* in CaP cells. Furthermore, we validated that *miR-125b* directly targets p53, Puma, and Bak1, which provide new insights into the molecular basis of CaP progression. There is little doubt that both p53 and AR play important roles in the progression of CaP. Here, we demonstrate that *miR-125b* links these two important pathways. Thus, it would appear to be an excellent target to pursue in our attempts to discover improved treatments for patients with CaP.

CONCLUSION

The present results demonstrate that overexpression of *miR-125b* promoted tumor growth in intact and castrated male nude mice. A series of experiments was performed to investigate the molecular mechanisms accounting for *miR-125b*-mediated growth of CaP cells. It was found that *miR-125b* down-regulates three key pro-apoptotic molecules: p53, Puma, and Bak1. Furthermore, reduction of *miR-125b* activity by using anti-

miR-125b oligonucleotides induced apoptosis of CaP cells and sensitizes CaP cells to anti-cancer drugs. These results suggest that *miR-125b* is oncogenic and makes it an attractive therapeutic target in prostate cancer.

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Down-Regulation of microRNA 106b Is Involved in p21-Mediated Cell Cycle Arrest in Response to Radiation in Prostate Cancer Cells

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BACKGROUND. microRNAs (miRNAs) are endogenous short non-coding RNAs, and play a pivotal role in regulating of a variety of cellular processes, including proliferation and apoptosis, both of which are cellular responses to radiation treatment. The purpose of this study is to identify candidate miRNAs whose levels are altered in response to radiation in prostate cancer cells and to investigate the molecular pathway of such miRNAs in the regulation of radiation-induced cellular response.

METHODS. Using a miRNA microarray assay, we screened 132 cancerous miRNAs in LNCaP cells in response to radiation treatment. The function of one candidate miRNA was investigated for checkpoint protein expression, cell cycle arrest, cell proliferation, and cell survival in cells transfected with precursor or antisense miRNA.

RESULTS. In response to radiation, multiple miRNAs, including mi-106b, showed altered expression. Cells transfected with precursor miR-106b were able to suppress radiation-induced p21 activation. Functionally, exogenous addition of precursor miR-106b overrode the G2/M arrest in response to radiation and resulted in a transient diminishment of radiation-induced growth inhibition.

CONCLUSION. We have shown a novel role of miR-106b, in the setting of radiation treatment, in regulating the p21-activated cell cycle arrest. Our finding that miR-106b is able to override radiation-induced cell cycle arrest and cell growth inhibition points to a potential therapeutic target in certain prostate cancer cells whose radiation resistance is likely due to consistently elevated level of miR-106b. *Prostate 71: 567–574, 2011.*

KEY WORDS: **microRNA; radiation; prostate cancer; cell cycle arrest**

INTRODUCTION

As the most common cancer among men in the United States, carcinoma of the prostate presents as a localized disease in 90% of known cases. For localized prostate cancer, both the American Urological Association and the National Comprehensive Cancer Network expert panels recommend radiation therapy either alone, or in combination with androgen deprivation therapy, as one of the main treatment modalities given its effectiveness for local cancer control [1,2].

Cancer killing effects after radiation treatment resulted from DNA double-strand breaks and other cellular injuries. Cellular responses to radiation-induced DNA damage are tightly controlled by cell cycle checkpoints. In response to radiation-induced DNA damage, cells are arrested at the transition from

Additional supporting information may be found in the online version of this article.

Abbreviations: miRNA, microRNA; XRT, external beam radiation.

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G1 to S phase (G1/S) or G2 to M phase (G2/M) of the cell cycle to allow DNA repair and cell survival. However, when damage is either unrepairable or misrepaired, cell death occurs through both mitotic and apoptotic processes [3,4]. Recently, considerable effort has been devoted to understanding the molecular mechanism of cell cycle arrest and its effect on cell death. The G1/S arrest results, at least in part, from p53-regulated synthesis of a cell cycle inhibitor p21 (CDKN1A), which leads to inhibition of the cyclin-cdk complex required for the transition from G1 to S phase [5,6]. Arrest in G2 phase after DNA damage is initiated by 14-3-3σ, a protein that controls activation of CHK1 and the cytoplasmic sequestration of the cyclinB-cdc2 complex which normally induces mitosis upon nuclear entry [7]. More importantly, recent evidence demonstrated that G2 phase arrest is sustained by p21 and p53 through degradation of anaphase-promoting complex (APC) [8–10].

In addition to being a target molecule of p53, p21 was recently shown to be inhibited at the post-transcriptional level by miR-106b, one of the microRNAs (miRNAs). This resulted in impaired function of p21 in the regulation of cell cycle arrest and apoptosis in response to TGF-β treatment in gastric cancer cells or Doxorubicin treatment in breast cancer cells [11,12]. Such miRNA regulation is mediated by a direct binding of miR-106b at two binding sites at the 3′-UTR of p21 mRNA [11,12]. These findings are just few of the rapidly emerging evidence that miRNAs, a group of endogenous short non-coding RNA molecules capable of silencing gene expression at the post-transcriptional level, play a pivotal role in regulation of cell proliferation, apoptosis, and tumorigenesis [13]. Based on the fact that cell proliferation is inhibited and cell death is triggered in response to radiation-induced DNA damage, we hypothesize that certain miRNAs could be involved in these cellular processes after radiation treatment. The purpose of this study is to identify candidate miRNAs whose levels are altered in response to radiation treatment in LNCaP prostate cancer cells and to investigate the molecular pathways in the regulation of radiation-induced cellular responses such as cell cycle arrest, cell proliferation inhibition, or cell death.

MATERIALS AND METHODS

Cell Culture

The prostate cancer cell lines LNCaP and MDA PCa 2b were maintained in RPMI medium supplemented with 10% fetal bovine serum, L-glutamine, vitamins, and penicillin and streptomycin. Cells were incubated at 37°C in 95% air/5% CO_2.

microRNA Array

Using TRIZOL reagent (Invitrogen Life Technology), total RNA was extracted from LNCaP cells treated with or without radiation. Total RNA was subjected to the miRNA array containing 132 cancerous miRNAs. A list of the 132 cancerous miRNAs is provided in Supplementary Table I. The assay was performed by a service provider (Signosis, Inc., Sunnyvale, CA). Results were detected with chemiluminescence and signals were acquired with the CCD camera-based imager system (Alpha Innotech). The signals of each spot were converted to density numbers with analysis software. For a specific miRNA, at least one of the samples should give an absolute density number greater than 15. After averaging two duplicate spots of each target, background subtraction, and normalization with RNU48, these arrays were compared side-by-side to identify differentially expressed miRNAs.

Cell Irradiation

Radiation treatments were performed as previously described [14]. Briefly, cells were irradiated at room temperature in the Department of Radiation Oncology in our institute, using a 15 MeV linear accelerator (Elekta, Stockholm, Sweden) with a source to bolus distance of 100 cm and a 20 × 16 cm field. The culture dishes were placed on top of a 5-cm solid water block (Gammes RI, Middleton, WI) for build up of the radiation dose and treated from 180° (posterior to anterior) with 6 Gy, at a dose rate of 2.5 Gy/min. The cells were returned to an incubator after the irradiations and maintained at 37°C until treated further or harvested.

cDNA Synthesis

Using TRIZOL reagent (Invitrogen Life Technology), total RNA was extracted from LNCaP cells treated with or without radiation. Samples were reverse transcribed using TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA). As per manufacturer's instruction, reactions were performed at 16°C for 30 min, followed by 42°C for 30 min. The enzymes were inactivated at 95°C for 5 min, and the reactions were subsequently used for real-time PCR.

Quantitative Real-Time PCR

The reaction containing cDNA was subsequently transferred for real-time PCR reaction for miR-106b amplification using TaqMan microRNA Assays kit (Applied Biosystems) as per manufacturer's instruction. As a control, primer for RNU6B was also used. The

real-time reactions of 15 μl were performed as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Each sample was run in triplicate. Complete TaqMan analysis was done using a 384-well laser 7900 HTA FAST platform (Applied Biosystems) in Molecular & Diagnostic Core Facility in our institute. As per manufacturer's instruction, the raw CT data were processed using the comparative CT method for relative gene transcription. The fold changes were depicted.

Transient Transfection

For analysis of miRNA interference, transient transfection was performed using the Lipofectamine 2000 kit as per manufacturer's instruction (Invitrogen) as previously described [15]. Briefly, 3×10^5 LNCaP cells were seeded overnight into 60-mm culture dishes containing in Lipo-Optim medium (Invitrogen). Precursor or antisense miR-106b (Pre-M or Anti-M, respectively) or their scrambled miRNAs (Pre-M-S or Anti-M-S) were obtained from Ambion (Austin, TX). Sixty nanomolars of respective miRNA was mixed with Lipofectamine 2000 (Invitrogen), and subsequently added to the medium. Anti-p21 siRNA (100 nmol/L; Pharmacon) was used as a control as previously described [15]. Cells were incubated in transfection mixture for 6 hr prior to termination of reaction by medium change. Cells were then incubated for 16 hr in regular medium prior to radiation treatment.

Western Blotting

Whole cell lysates were prepared at the time indicated. Western blot analysis was performed as previously described [15]. Primary antibodies included anti-p21, and anti-p53, (Santa Crux Biotechnology). Densitometry analysis was performed using Quantity One software. Protein levels were normalized to β-actin, and fold changes were determined and plotted.

Flow Cytometry

Flow cytometry was performed as previously described [15]. Briefly, cells were trypsinized, washed with PBS, resuspended in 70% ethanol, and kept at −20°C overnight. Cells were subsequently centrifuged, washed in PBS, resuspended in 450 μl of PBS and 10 μl of 10 μg/ml DNase-free RNase (Roche), and then incubated at 37°C for 45 min. Following RNase treatment, 50 μl of propidium iodine (Boehringer Mannheim Corp.) was added, and cells were incubated at room temperature for 10 min with light protection. Cell aggregates were removed by filtration prior to analysis. Cell cycle analysis was done in the Coulter Epics XL flow cytometer (Beckman Coulter). The population of cells in each of the sub-G1, G1, S, and G2 phases were determined on at least 250,000 cells with doublet discrimination. Analysis of cell cycle position was done using the Phoenix Multicycle software (Phoenix Flow Systems).

Cell Growth Assay

Cells were transfected and irradiated as described above. At different time points post-radiation, cell growth was detected using the WST-1 assay (Roche) as per manufacturer's instructions. Briefly, cells were incubated in reaction buffer for 20 min prior to the addition of WST-1 solution. Cells were detected at 500 nM on a Beckman plate reader (Beckman Coulter).

Clonogenic Assay

Clonogenic assay was performed as previously described [15]. Briefly, cells were transfected as described above with the exception that cells were replated as single cells at 24 hr post-transfection with 4×10^4 cells per 60-mm plate. At 24 hr after replating, cells were irradiated. Cells were cultured for 12 days (approximately 6 doubling times) prior to fixation and staining in 1.0% crystal violet in 0.5% glacial acetic acid in ethanol. Surviving colonies containing approximately 50 or more cells were counted.

Statistical Analysis

Using the JMP statistical software package (SAS Corporate, Cary, NC), Student's *t*-test was performed to determine the statistical significance compared with the control or compared between groups ($^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$). All experiments were performed in triplicate, repeated three times. Representative findings were shown.

RESULTS

p53 and p21 Activation Is Associated With G1/S and G2/M Cell Cycle Arrest in Response to Radiation Treatment

Figure 1 shows that in LNCaP cells, 6 Gy external beam radiation (XRT) caused remarkable elevation of the G2 cell population, indicating G2/M arrest. In spite of a mild elevation of G1 population after radiation, the lack of S phase suggested a presence of G1/S arrest. Radiation induced G1/S and G2/M cell cycle arrest started 6 hr after treatment and reached a plateau at 15 and 24 hr. The cell cycle arrest was associated with elevated protein level of p53 and p21. However, the increase of p53 protein level was not as remarkable as that of p21.

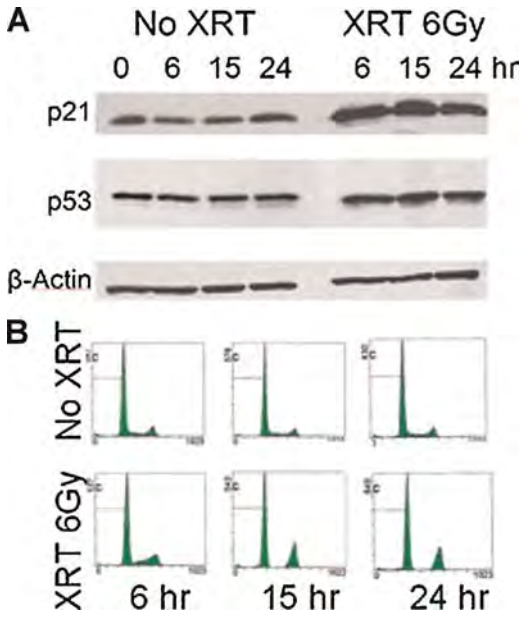

Fig. 1. Activation of p21, p53, and cell cycle arrest induced by radiation in LNCaP cells. **A**: Western blotting of p21 and p53 at different time points after 6 Gy of radiation. **B**: Representative cell cycle profiles of cells fixed and subject to flow cytometry at different time points after 6 Gy of radiation.

Differential Expression of miRNA in Prostate Cancer Cells in Response to Radiation Treatment

With the intent to identify miRNA candidates that may contribute to the radiation induced-G2/M cell cycle arrest, microarray analysis of 132 cancerous miRNAs was performed using RNA from LNCaP cells collected 24 hr post-radiation. This miRNA profile was compared to that from cells that did not receive radiation. Table I shows a summary of miRNAs whose expression was changed in response to radiation. Among them miR-106b demonstrated the most significant suppression. This finding was further confirmed in quantitative real-time PCR which revealed a threefold suppression of miR-106b at 24 and 48 hr post-radiation when LNCaP cells undergo cell cycle arrest. The result suggests a physiological response to radiation (Fig. 2).

TABLE I. miRNAs With Altered Expression in Response to Radiation in LNCaP Cells Using miRNA Microarray Assay

microRNA	miR change (in fold) in LNCaP cells
has-miR-106b	3.3 (decrease)
has-miR-199a	1.4 (decrease)
has-miR-29b	7.3 (increase)
has-miR-191	3.3 (increase)
has-miR-22	2.7 (increase)
has-miR-200c	2.7 (increase)
has-miR-141	2.3 (increase)
has-miR-24	2.3 (increase)
has-miR-30a-5p	2.2 (increase)
has-miR-9-1	1.4 (increase)

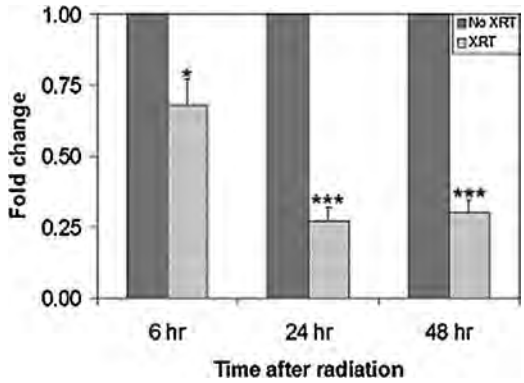

Fig. 2. Quantitative real-time PCR analysis of miR-106b in LNCaP cells at different time points after 6 Gy of radiation treatment. miR-106b expression was normalized to RNU6B. Fold change is the ratio of miR-106b in cells treated with radiation/without radiation. $*P < 0.05$; $***P < 0.001$.

Transfected Precursor miR-106b Suppressed the Radiation-Induced p21 Activation

miR-106b was chosen for further analysis based on published evidence that the cell cycle inhibitor p21/CDKN1A is the direct target of miR-106b, and is the key regulator for cell cycle arrest in response to radiation-induced DNA damage. Using a transient transfection assay, we introduced precursor or antisense miR-106b into LNCaP cells with intent to either overexpress or suppress the endogenous miR-106b down-regulated by radiation. We showed that the ectopically introduced precursor miR-106b, but not antisense miR-106b or the scrambled miRNA negative controls, was able to suppressed the radiation-induced elevation p21 at 24 hr post-treatment (Fig. 3). Of note, the synthetic pre-miR-106b is a short single-stranded RNA oligonucleotide that has been chemically modified to increase precursor stability and miR-106b activity in vivo. The anti-miR-106b is a double-stranded RNA oligonucleotide designed to specifically bind to endogenous miR-106b, inhibiting miR-106b activity but not down-regulating its abundance. As a control, anti-p21 siRNA successfully down-regulated p21 protein to its basal level after irradiation. Western blot results using MDA PCa 2b cells confirmed the above results in LNCaP cells (Supplementary Fig. 1).

Precursor miR-106b Overrode Radiation-Induced G2/M Arrest, But Not G1/S Arrest

To determine the functional role of miR-106b in p21-dependent cell cycle arrest, flow cytometry analysis

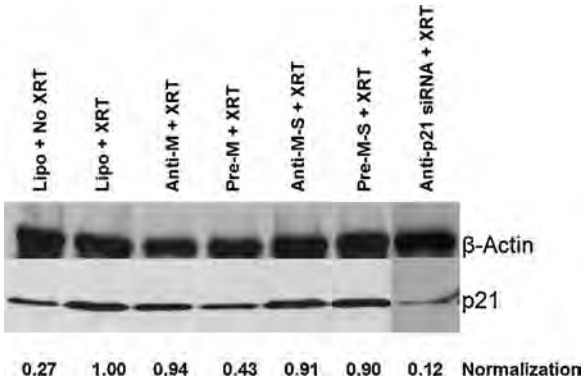

Fig. 3. Ectopically introduced precursor miRI06b (Pre-M) suppressed radiation-induced p2I activation. The intensity of the bands was quantified using Quantity One software and was normalized to that of β-actin. Anti-M: antisense miR-I06b; Anti-M-S and Pre-M-S: scrambled sequences.

was performed in LNCaP cells transfected with precursor or antisense miR-106b and subsequently treated with radiation. Flow cytometry showed that exogenous addition of pre-miR-106b, but not anti-miR-106b, resulted in partial override of radiation-induced G2/M arrest (Fig. 4A), suggesting that miR-106b gain of function is sufficient to override a radiation induced G2/M checkpoint. However, the absence of S phase recovery in pre-miR-106b transfected cells suggested the lack of a G1/S phase arrest override. Anti-p21 siRNA caused a more remarkable reduction of the radiation-induced G2/M peak than pre-miR-106b, but did not completely normalize the radiation-induced G2/M peak. Flow cytometry results using MDA PCa 2b cells confirmed the above results in LNCaP cells (Supplementary Fig. 2).

Precursor miR-106b Caused Diminished Radiation-Induced Cell Growth Inhibition

With the intent to further determine the impact on cell proliferation as a result of the override of G2/M arrest, WST-1 cell viability assay was performed at different time points post-radiation treatment of transfected LNCaP cells. At post-radiation day 1 and day 3, a statistically significant abolishment of the radiation-induced cell growth inhibition was observed in cells

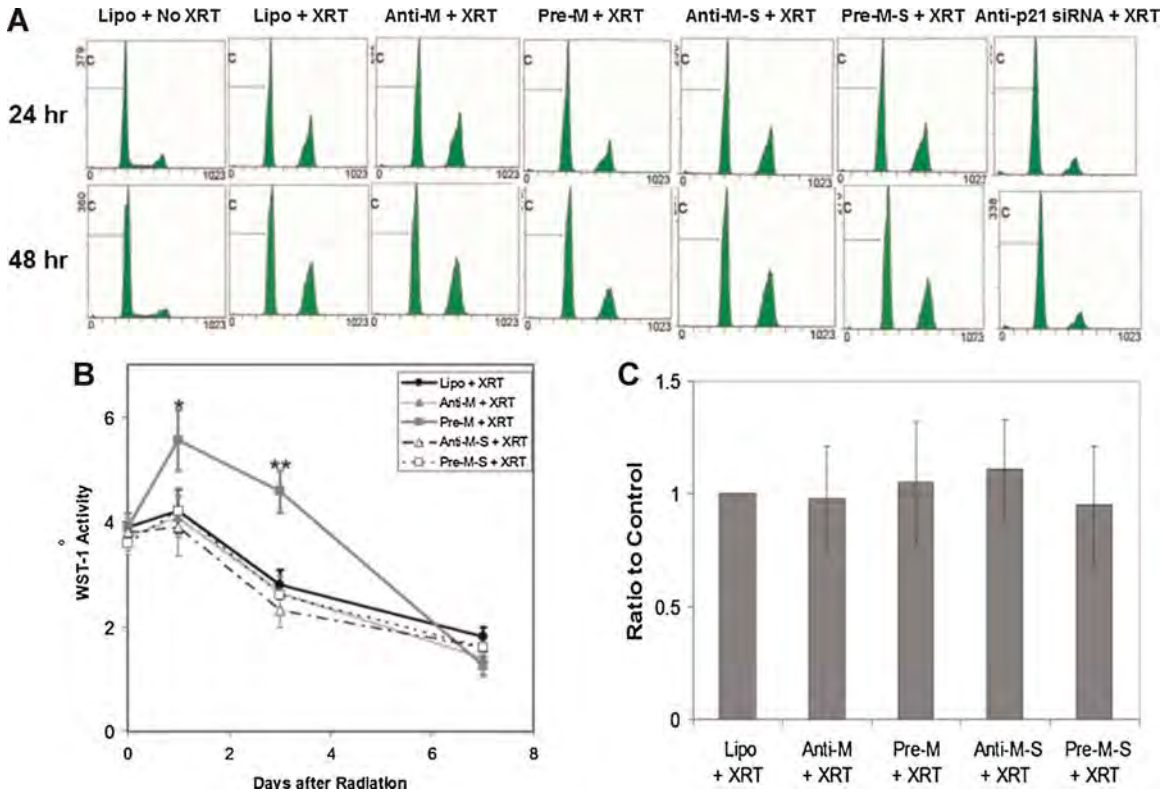

Fig. 4. Functional analysis of LNCaP cells with exogenous addition of precursor (Pre-M) or antisense (Anti-M) miR-I06b. **A**: Representative cell cycle profiles of cells transfected with miR-I06b mimics and subject to flow cytometry at different time points after 6 Gy of radiation. **B**: Cell growth curves of cells transfected with miR-I06b mimics followed by 6 Gy of radiation and subject to WST-I assay. **C**: Analysis of cell survival ability by clonogenic assay of cells transfected with miR-I06b mimics followed by 6 Gy of radiation. Cells were cultured for I2 days prior to colony counting. $^{*}P < 0.05$; $^{**}P < 0.005$. Anti-M-S and Pre-M-S: scrambled sequences. All experiments were performed in triplicate, repeated three times in separate studies. Representative findings were shown.

transfected with precursor miR-106b (Fig. 4B). Nevertheless, this diminished cell growth arrest did not last for more than 1 week after radiation. Furthermore, clonogenic assay revealed lack of statistically significant difference in clone formation among LNCaP cells transfected with pre-miR-106b, anti-miR-106b or the scrambled miR-106b (Fig. 4C). This indicated that the early increase in cell proliferation did not translate into long term clonogenic survival capacity.

DISCUSSION

It is a well-studied observation that, in response to radiation-induced DNA damage, cells are arrested at the G1/S or G2/M phase of the cell cycle through mechanisms involving p53, p21, cyclin/cdks, and other checkpoint molecules. Taking it further, the present study is the first to document the novel role of a specific miRNA, namely miR-106b, in response to radiation treatment. Moreover, we showed that in irradiated LNCaP cells the regulatory functions of miR-106b in G2/M cell cycle arrest and cell proliferation inhibition are mediated by p21, an established direct target of miR-106b.

In line with our findings, Petrocca et al. [11] demonstrated that the miR-106b suppression was involved in the regulation of TGF-β induced G1/S arrest and is partially responsible for cell viability in gastric cancer cells. In addition, in breast cancer cells, overexpression of miR-106b directly inhibited p21 and promoted progression through Doxorubicin-induced G1/S and G2/M cell cycle arrest [12]. In spite of the established role of miR-106b in p21-mediated cell cycle arrest, the location of the checkpoint in cell cycle remains unclear. Our study suggested an involvement of miR-106b in the G2/M arrest, but not the G1/S arrest. However, results from other studies indicated that miR-106b acted at the G1/S transition in addition to the G2/M arrest [11,12]. This discrepancy is likely due to the difference in treatment and/or cell lines investigated. In the present study, we used X-ray radiation for LNCaP prostate cancer cells, rather than Doxorubicin or TGF-β treatment for breast cancer or gastric cancer cells as described in the studies mentioned above.

The radiation-induced suppression of miR-106b and its role in cell cycle regulation are of clinical importance for radiation therapy which is one of the two major treatment modalities for early stage prostate cancer. Conventional XRT therapy is delivered with 2 Gy per fraction in 35–40 daily fractions. Multi-fraction radiation therapy takes advantage of the reassortment process to improve efficacy: after each daily radiation, cells progress through S phase (the least radiosensitive) and are arrested in G2/M phase (the most radiosensitive) or G1/S phase (intermediate sensitive) in time for the next daily treatment. Such an effect has been confirmed in vitro using human prostate cell lines: (1) doses of 2 or 4 Gy radiation lead to a shift towards a predominance of cells in G2/M phase, causing the prostate cells to be more sensitive to radiation [16]; (2) in LNCaP cells, the change of cell cycle distribution following fractionated radiation was predictive of ultimate cell survival response [14].

Although mild, our finding of a threefold suppression of miR-106b after radiation is of clinical significance. This is supported by data from Ambs et al. [17] that miR-106b expression was elevated threefold in primary prostate tumors, compared to normal prostate tissue. Similarly, miR-106b has been found to be twofold higher in gastric adenocarcinoma when compared to normal gastric mucosa [11]. In addition, our data suggest that miR-106b could be of a potential clinical target for early stage patients who developed biochemical failure after definitive radiation therapy. Multiple studies have demonstrated 10–20% biochemical failure at 10 years after treatment [1]. It is possible that in certain prostate cancer cells, radiation resistance is in part due to the abnormally elevated level of miR-106b which is not suppressed by radiation therapy. It is supported by the data from Ambs et al. [17] that miR-106b expression was elevated in primary prostate tumors. In this setting, miR-106b could be of a potential therapeutic target. However, our result did not show further elevation of p21 levels or cell cycle arrest by transfected anti-miR-106, and thus such a hypothesis needs further experimental investigation. A possible explanation for the lack of change in p21 levels is that miR-106 level is maximally suppressed by radiation in LNCaP cells. Therefore, addition of anti-miR-106b causes no further decrease in miR-106b levels or any subsequent change in p21 levels or cell cycle arrest.

The mechanism of p21 suppression by miR-106b has been recently illustrated: p21 is a direct target of miR-106b via the seed region hexamer complements in the p21 mRNA 3′-UTR [11,12,17]. Furthermore, published data from our laboratory showed that when p21 was silenced with an anti-p21 siRNA, the G2/M population was significantly abolished in LNCaP cells treated with 10 Gy of radiation [15]. These two lines of evidence strongly support our conclusion that the involvement of miR-106b in G2/M cell cycle arrest is mediated by its direct inhibition of p21. The present data confirmed that p21 silencing by anti-p21 siRNA caused a significant reduction of the radiation-induced G2/M peak. Notably, the G2/M peak reduction is more remarkable using anti-p21 siRNA than pre-miR-106b. However, the anti-p21 siRNA did not completely normalize the radiation-induced G2/M peak. This

observation indicates potential novel pathways of miR-106b in radiation response and cell cycle regulation. A search of the TargetScanHuman database identified more than 900 putative miR-106b target genes, including p21 gene [18]. Some other targets of potential significance are genes involved in cell cycle regulation (Rb-like 1 and Rb-like 2) and apoptosis (caspase 2 and capase 7). Further studies are underway to investigate these potential novel pathways of miR-106b.

miR-106b, alone with miR-93 and miR-25, is one of the three highly conserved miRNAs within the miR-106b-25 cluster located in a 515-bp region at chromosome 7q22, in intron 13 of the host gene Mcm7 [19]. The three members of the cluster are cotranscribed in the context of the primary transcript of Mcm7, the overexpression of which has been associated with bad prognosis in prostate cancer [20]. Based on the finding that these miRNAs are accumulated in different cancer types, they are emerging as an important component of cellular networks relevant to oncology [19]. In particular, all members of the miR-106b-25 cluster are highly expressed in primary prostate tumor, compared to normal prostate tissue [17]. In our study, the microarray profile did not identify a change of miR-93 and miR-25 expression in response to radiation, indicating a regulation of primary miRNAs at post-transcriptional level. This differential expression is supported by findings that only miR-106b, but not miR-93 or miR-25, is functionally involved cell cycle arrest [12]. Of more significance, Petrocca et al. showed different functions of the three members of the miR-106b-25 cluster: whereas miR-106b and miR-93 suppress p21 expression that is required for TGF-β-induced cell cycle arrest, miR-25 silences BCL2L11 (BIM) expression that is essential for TGF-β-dependent apoptosis [19].

Besides miR-106b, we also identified several other important miRNAs including miR-141 and miR-9-1, whose expressions were elevated in response to radiation. A pioneering work by Mitchell et al. [21] showed that the serum level of miR-141 was able to serve as a circulating biomarker for distinguishing prostate cancer patients from healthy controls. Laios et al. [22] found that miR-9-1 was pro-apoptotic, with bcl-2 as one of its putative target genes. In metastatic ovarian or brain cancers, the level of miR-9-1 was significantly inhibited when compared with its level in tumors at primary site [22]. The biological function of the two miRNAs in response to radiation will be the object of our future study. Surprisingly, our study did not find any changes in the p53-induced miR-34. One possible explanation is different levels of miR-34 activation at different time points after irradiation. It was demonstrated by He et al. [23] that miR-34 level was elevated to 10-fold at 4 hr after irradiation with a dose of 6 Gy, but was 4-fold at 8 hr after irradiation. Given that our samples were collected at 24 hr after irradiation, it is possible that elevation of miR-34 may not have been sustained long enough to be detected in our study.

In addition to our findings, only one other study has investigated altered expression of miRNAs in response to radiation in prostate cancer cells [24]. Josson et al. demonstrated a different group of miRNAs at 4 hr after 6 Gy of radiation treatment in LNCaP cells. Among these miRNAs is miR-521, whose expression was down-regulated by radiation, with its target being a DNA repair protein Cockayne syndrome protein A. The discrepancy in the miRNA profile is likely due to the time dependence of miRNA expression post radiation, given that our miRNA pattern was obtained at 24 hr post-radiation.

One of the inherent limitations is the cell culture nature of this study. First of all, some of our results using LNCaP cells were confirmed in MDA PCa 2b cells, but not in other cell lines such as PC-3 or CWR22R. This is likely due to heterogeneous molecular characterizations among these prostate cell lines such as p53 loss of heterozygosity, nucleotide mutation, different expression level, neuroendocrine differentiation, and cytogenetics. Given the well-established central role of p53 in cellular response to radiation-induced DNA damage, it is not surprising that neither LNCaP nor MDA PCa 2b cell lines contains any p53 loss of heterozygosity or nucleotide mutation while both PC-3 and CWR22R do. Secondly, our results using transient transfection need to be further confirmed in animal models. Studies are underway to investigate the effect of miR-106b in animal models using lentivirus transfected prostate cancer cells. In addition, our observations suggested a biological context where regulation of multiple targets by a single miRNA (miR-106b) is able to exert cell cycle arrest. Further study is underway to investigate these potential novel pathways of miR-106b in radiation response and cell cycle regulation.

CONCLUSION

Given its effectiveness for local disease control, radiation therapy is one of the main modalities for treating early stage prostate cancer. While several mechanisms have been established which contribute to cell cycle arrest and likely cell growth inhibition, we have shown a novel role of miR-106b, in the setting of radiation treatment, in regulating the p21-activated cell cycle arrest. Our finding that miR-106b is able to override radiation-induced cell cycle arrest and cell growth inhibition points to a potential therapeutic target in certain radiation-resistant prostate cancer cells whose high level of miR-106b level is not suppressed by radiation therapy.

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